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Peter Andersen et al

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For: THERAPEUTIC TB VACCINE

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Sirs:

Attached please find the certified copy of the foreign application from
which priority is claimed in the above-identified U.S. Patent application:

Country:	Denmark
Application No.	PA 2000 00666
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Respectfully submitted,
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Title: Nucleic acid fragments and polypeptide fragments derived from M. Tuberculosis.

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This is to certify that the attached documents are exact copies of the above mentioned patent application as originally filed.



Patent- og Varemærkestyrelsen
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26 November 2003

Hgs
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19 APR. 2000

Nucleic acid fragments and polypeptide fragments derived from *M. Tuberculosis*

FIELD OF THE INVENTION

The present invention relates to immunologically active, polypeptide fragments derived
5 from the *Mycobacterium tuberculosis*, vaccines and other immunologic compositions
containing the fragments as immunogenic components, and methods of production and
use of the polypeptides. The invention also relates to novel nucleic acid fragments derived
from *M. tuberculosis* which are useful in the preparation of the polypeptide fragments of
the invention or in the diagnosis of infection with *M. tuberculosis*.

10

Background of the invention

Human tuberculosis (hereinafter designated "TB") caused by *Mycobacterium tuberculosis*
is a severe global health problem responsible for approximately 3 million deaths annually,
according to the WHO. The worldwide incidence of new TB cases has been progressively
15 falling for the last decade but the recent years has markedly changed this trend due to the
advent of AIDS and the appearance of multidrug resistant strains of *M. tuberculosis*.

The only vaccine presently available for clinical use is BCG, a vaccine which efficacy
remains a matter of controversy. BCG generally induces a high level of acquired
20 resistance in animal models of TB, but several human trials in developing countries have
failed to demonstrate significant protection. Notably, BCG is not approved by the FDA for
use in the United States. Although the BCG vaccine is widely used, some countries
including the USA never introduced it for use in general population vaccination
programmes, one reason being that vaccination with BCG interferes with the use of
25 tuberculin skin testings for diagnosing tuberculosis and for use in population surveys.

This makes the development of a new and improved vaccine against TB an urgent matter
which has been given a very high priority by the WHO.

30 It is an object of the invention to provide novel antigens which are effective as
components in a subunit vaccine against TB or which are useful as components in
diagnostic compositions for the detection of infection with mycobacteria, especially

virulence-associated mycobacteria. The novel antigens may also be important drug targets.

Summary of the invention

Name of antigen	Nucleotide sequence SEQ ID NO:	Amino acid sequence SEQ ID NO:
Rv0284	1	2
Rv0284 3' part / c-terminal	3	4
Rv0285	5	6
Rv3878 (ORF11)	7	8

5

Detailed disclosure of the invention

The present invention is i.a. based on the identification and characterisation of a number of previously uncharacterised antigens from *M. tuberculosis* as presented in the

10 examples. It is well-known in the art that T-cell epitopes are responsible for the elicitation of the acquired immunity against TB. Since such T-cell epitopes are linear and are known to have a minimum length of 6 amino acid residues, the present invention is especially concerned with the identification and utilisation of such T-cell epitopes.

15 As illustrated in example 2, Rv0284 (SEQ ID NO: 2) causes a marked release of IFN- γ from PBMC withdrawn from TB patients from half of the donors, and in a majority of PPD positive healthy donors without any increase in PPD negative healthy donors. Rv0284 further stimulates T cell lines from PPD positive donors to release IFN- γ and induces a DTH reaction in guinea pigs aerosol infected with *M. tuberculosis*. This indicates that

20 Rv0284 is highly biologically active and recognised by PPD positive donors and TB patients.

Thus, one aspect of the invention relates to a substantially pure polypeptide fragment which comprises an amino acid sequence as shown in SEQ ID NO: 2 or comprises an

25 amino acid sequence analogue having a sequence identity with the polypeptide fragment shown in SEQ ID NO: 2 of at least 70% and at the same time being immunologically equivalent to the polypeptide fragment shown in SEQ ID NO: 2

A related aspect of the invention relates to a substantially pure polypeptide fragment which comprises a T-cell epitope of the amino acid sequence as shown in SEQ ID NO: 2 and at the same time being immunologically equivalent to the polypeptide shown in SEQ ID NO: 2.

As illustrated in example 2, Rv0285 (SEQ ID NO: 6) stimulates T cell lines from PPD positive donors to release IFN- γ to a level of close to the release caused by PPD. Rv0285 further induces a DTH reaction in guinea pigs aerosol infected with M. tuberculosis. This indicates that Rv0285 is highly biologically active and recognised by PPD positive donors and TB patients.

As illustrated in example 2, Rv3878 (SEQ ID NO: 8) causes a marked release of IFN- γ from PBMC withdrawn from TB patients and in PPD positive healthy donors without any increase in PPD negative healthy donors. Rv3878 further stimulates T cell lines from PPD positive donors to release IFN- γ to a level resembling the release caused by PPD and Rv0285 also induces a DTH reaction in guinea pigs aerosol infected with M. tuberculosis.. This indicates that Rv3878 is highly biologically active and recognised by PPD positive donors and TB patients.

20

A polypeptide fragment is considered to be "immunologically equivalent" to a polypeptide disclosed in the present invention, if it

- 1) induces *in vitro* recall response determined by release of IFN- γ of at least 30% of the release induced by the polypeptide disclosed from Peripheral Blood Mononuclear Cells (PBMC) or whole blood withdrawn from TB patients 0-6 months after diagnosis, or PPD positive individual, the inductions being performed by the addition of the polypeptide disclosed and the polypeptide fragment to two individual suspensions comprising about 1.0 to 2.5×10^5 PBMC or whole blood cells, the addition of the polypeptides resulting in a concentration of not more than $20 \mu\text{g}$ per ml suspension, the release of IFN- γ being assessable by determination of IFN- γ in supernatant harvested 5 days after the addition of the polypeptide to the suspension; or
- 2) it induces a positive DTH response determined by intradermal injections or local application patches of at most $100 \mu\text{g}$ of the polypeptide disclosed and of the polypeptide fragment to an individual who is clinically or subclinically infected with a virulent *Mycobacterium*, and the polypeptide fragment causing a response diameter that is at least

50% of that caused by the polypeptide disclosed measured 72-96 hours after the injections or applications.

- 5 Each polypeptide disclosed in the present application is characterised by specific amino acid and nucleic acid sequences. It will be understood that such sequences include analogues and variants produced by recombinant methods wherein such nucleic acid and polypeptide sequences have been modified by substitution, insertion, addition and/or deletion of one or more nucleotides in said nucleic acid sequences to cause the
- 10 substitution, insertion, addition or deletion of one or more amino acid residues in the recombinant polypeptide.

In both immunodiagnostics and vaccine preparation, it is often possible and practical to prepare antigens from segments of a known immunogenic protein or polypeptide. Certain

15 epitopic regions may be used to produce responses similar to those produced by the entire antigenic polypeptide. Potential antigenic or immunogenic regions may be identified by any of a number of approaches, e.g., Jameson-Wolf or Kyte-Doolittle antigenicity analyses or Hopp and Woods (1981) hydrophobicity analysis (see, e.g., Jameson and Wolf, 1988; Kyte and Doolittle, 1982; or U.S. Patent No. 4,554,101). Hydrophobicity

20 analysis assigns average hydrophilicity values to each amino acid residue from these values average hydrophilicities can be calculated and regions of greatest hydrophilicity determined. Using one or more of these methods, regions of predicted antigenicity may be derived from the amino acid sequence assigned to the polypeptides of the invention.

- 25 Alternatively, in order to identify relevant T-cell epitopes which are recognized during an immune response, it is also possible to use a "brute force" method: Since T-cell epitopes are linear, deletion mutants of polypeptides having SEQ ID NO: 2, 4, 6, or 8 will, if constructed systematically, reveal what regions of the polypeptides are essential in immune recognition, e.g. by subjecting these deletion mutants to the IFN- γ assay described herein.
- 30 Another method utilises overlapping oligomers (preferably synthetic having a length of e.g. 20 amino acid residues) derived from polypeptides having SEQ ID NO: 2, 4, 6, or 8. Some of these will give a positive response in the IFN- γ assay whereas others will not.

In a preferred embodiment of the invention, the polypeptide fragment of the invention

35 comprises an epitope for a T-helper cell.

Although the minimum length of a T-cell epitope has been shown to be at least 6 amino acids, it is normal that such epitopes are constituted of longer stretches of amino acids. Hence it is preferred that the polypeptide fragment of the invention has a length of at least 7 amino acid residues, such as at least 8, at least 9, at least 10, at least 12, at least 14, at least 16, at least 18, at least 20, at least 22, at least 24, and at least 30 amino acid residues.

By producing fusion polypeptides, superior characteristics of the polypeptide fragments of the invention can be achieved. For instance, fusion partners which facilitate export of the polypeptide when produced recombinantly, fusion partners which facilitate purification of the polypeptide, and fusion partners which enhance the immunogenicity of the polypeptide fragment of the invention are all interesting possibilities. Therefore, the invention also pertains to a fusion polypeptide comprising at least one polypeptide fragment defined above and at least one fusion partner. The fusion partner can, in order to enhance immunogenicity, e.g. be selected from the group consisting of another polypeptide fragment as defined above (so as to allow for multiple expression of relevant epitopes), and an other polypeptide derived from a bacterium belonging to the tuberculosis complex, such as ESAT-6, MPB64, MPT64, and MPB59 or at least one T-cell epitope of any of these antigens. Other immunogenicity enhancing polypeptides which could serve as fusion partners are T-cell epitopes (e.g. derived from the polypeptides ESAT-6, MPB64, MPT64, or MPB59) or other immunogenic epitopes enhancing the immunogenicity of the target gene product, e.g. lymphokines such as INF- γ , IL-2 and IL-12. In order to facilitate expression and/or purification the fusion partner can e.g. be a bacterial fimbrial protein, e.g. the pilus components pilin and papA; protein A; the ZZ-peptide (ZZ-fusions are marketed by Pharmacia in Sweden); the maltose binding protein; glutathione S-transferase; β -galactosidase; or poly-histidine.

Other interesting fusion partners are polypeptides which are lipidated and thereby effect that the immunogenic polypeptide is presented in a suitable manner to the immune system. This effect is e.g. known from vaccines based on the *Borrelia burgdorferi* OspA polypeptide, wherein the lipidated membrane anchor in the polypeptide confers a self-adjuvating effect to the polypeptide (which is natively lipidated) when isolated from cells producing it. In contrast, the OspA polypeptide is relatively silent immunologically when prepared without the lipidation anchor.

A substantially pure polypeptide according to any of the preceding claims for use as a pharmaceutical.

5 Use of a substantially pure polypeptide according to any of the preceding claims for the preparation of a pharmaceutical composition for the diagnosis of tuberculosis caused by *Mycobacterium tuberculosis*, *Mycobacterium africanum* or *Mycobacterium bovis*.

10 Use of a substantially pure polypeptide according to any of the preceding claims for the preparation of a pharmaceutical composition for the vaccination against infections caused by *Mycobacterium tuberculosis*, *Mycobacterium africanum* or *Mycobacterium bovis*.

15 Another part of the invention pertains to an immunologic composition comprising a polypeptide or fusion polypeptide according to the invention. In order to ensure optimum performance of such an immunologic composition it is preferred that it comprises an immunologically and pharmaceutically acceptable carrier, vehicle or adjuvant.

Suitable carriers are selected from the group consisting of a polymer to which the polypeptide(s) is/are bound by hydrophobic non-covalent interaction, such as a plastic, e.g. polystyrene, or a polymer to which the polypeptide(s) is/are covalently bound, such as a polysaccharide, or a polypeptide, e.g. bovine serum albumin, or keyhole limpet haemocyanin. Suitable vehicles are selected from the group consisting of a diluent and a suspending agent. The adjuvant is preferably selected from the group consisting of dimethyldioctadecylammonium bromide (DDA), Quil A, poly I:C, Freund's incomplete adjuvant, IFN- γ , IL-2, IL-12, monophosphoryl lipid A (MPL), and muramyl dipeptide (MDP).
25

A preferred immunologic composition according to the present invention comprising at least two different polypeptide fragments, each different polypeptide fragment being a polypeptide or a fusion polypeptide defined above. It is preferred that the immunologic composition comprises between 3-20 different polypeptide fragments or fusion polypeptides.
30

Such an immunologic composition may preferably be in the form of a vaccine or in the form of a skin test reagent.

In line with the above, the invention therefore also pertains to a method for producing an immunologic composition according to the invention, the method comprising preparing, synthesizing or isolating a polypeptide according to the invention, and solubilizing or dispersing the polypeptide in a medium for a vaccine, and optionally adding other M.
5 tuberculosis antigens and/or a carrier, vehicle and/or adjuvant substance.

Each of the polypeptides may be characterised by specific amino acid and nucleic acid sequences. It will be understood that such sequences include analogues and variants produced by recombinant methods wherein such nucleic acid and polypeptide sequences
10 have been modified by substitution, insertion, addition and/or deletion of one or more nucleotides in said nucleic acid sequences to cause the substitution, insertion, addition or deletion of one or more amino acid residues in the recombinant polypeptide. A preferred nucleotide sequence encoding a polypeptide of the invention is a nucleotide sequence which

15

1) is a DNA sequence selected from the group consisting of SEQ ID NOs: 1, 3, 5, and 7 or an analogue of said sequence which hybridises with any DNA sequence complementary to DNA sequences shown in SEQ ID NOs: 1, 3, 5, or 7 or a specific part thereof, preferably under stringent hybridisation conditions. By stringent conditions is understood,
20 as defined in the art, 5-10°C under the melting point T_m , cf. Sambrook et al, 1989, pages 11.45-11.49, and/or

2) encodes a polypeptide, the amino acid sequence of which has a 80% sequence identity with an amino acid sequence selected from the group consisting of SEQ ID NOs: 2, 4, 6,
25 and 8 and/or

3) constitutes a subsequence of any of the above mentioned DNA sequences, and/or

4) constitutes a subsequence of any of the above mentioned polypeptide sequences.
30

The terms "analogue" or "subsequence" when used in connection with the DNA fragments of the invention are thus intended to indicate a nucleotide sequence which encodes a polypeptide exhibiting identical or substantially identical immunological properties to a polypeptide encoded by the DNA fragment of the invention shown in any of SEQ ID NOs:
35 1, 3, 5, or 7, allowing for minor variations which do not have an adverse effect on the

ligand binding properties and/or biological function and/or immunogenicity as compared to any of the polypeptides of the invention or which give interesting and useful novel binding properties or biological functions and immunogenicities etc. of the analogue and/or subsequence. The analogous DNA fragment or DNA sequence may be derived from a
5 bacterium, a mammal, or a human or may be partially or completely of synthetic origin. The analogue and/or subsequence may also be derived through the use of recombinant DNA techniques.

Furthermore, the terms "analogue" and "subsequence" are intended to allow for variations
10 in the sequence such as substitution, insertion (including introns), addition, deletion and rearrangement of one or more nucleotides, which variations do not have any substantial effect on the polypeptide encoded by a DNA fragment or a subsequence thereof. The term "substitution" is intended to mean the replacement of one or more nucleotides in the full nucleotide sequence with one or more different nucleotides, "addition" is understood to
15 mean the addition of one or more nucleotides at either end of the full nucleotide sequence, "insertion" is intended to mean the introduction of one or more nucleotides within the full nucleotide sequence, "deletion" is intended to indicate that one or more nucleotides have been deleted from the full nucleotide sequence whether at either end of the sequence or at any suitable point within it, and "rearrangement" is intended to mean
20 that two or more nucleotide residues have been exchanged with each other.

It is well known that the same amino acid may be encoded by various codons, the codon usage being related, *inter alia*, to the preference of the organisms in question expressing the nucleotide sequence. Thus, at least one nucleotide or codon of a DNA fragment of the
25 invention may be exchanged by others which, when expressed, result in a polypeptide identical or substantially identical to the polypeptide encoded by the DNA fragment in question.

When the term nucleic acid is used in the following, it should be understood that for the
30 number of purposes where nucleic acid can be substituted with DNA or RNA, which will be apparent for the person skilled in the art. For the purposes of hybridization, PNA or LNA may be used instead of DNA or nucleic acid. As DNA is the most frequently used transfection material DNA is the preferred nucleic acid.

The invention also relates to a replicable expression vector which comprises a nucleic acid fragment defined above, especially a vector which comprises a nucleic acid fragment encoding a polypeptide fragment of the invention.

- 5 The vector may be any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication; examples of such a vector are a plasmid, phage, cosmid, mini-
10 chromosome or virus. Alternatively, the vector may be one which, when introduced in a host cell, is integrated in the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

- Expression vectors may be constructed to include any of the DNA segments disclosed
15 herein. Such DNA might encode an antigenic protein specific for virulent strains of mycobacteria or even hybridization probes for detecting mycobacteria nucleic acids in samples. Longer or shorter DNA segments could be used, depending on the antigenic protein desired. Epitopic regions of the proteins expressed or encoded by the disclosed DNA could be included as relatively short segments of DNA. A wide variety of expression
20 vectors is possible including, for example, DNA segments encoding reporter gene products useful for identification of heterologous gene products and/or resistance genes such as antibiotic resistance genes which may be useful in identifying transformed cells.

- The vector of the invention may be used to transform cells so as to allow propagation of
25 the nucleic acid fragments of the invention or so as to allow expression of the polypeptide fragments of the invention. Hence, the invention also pertains to a transformed cell harbouring at least one such vector according to the invention, said cell being one which does not natively harbour the vector and/or the nucleic acid fragment of the invention contained therein. Such a transformed cell (which is also a part of the invention) may be
30 any suitable bacterial host cell or any other type of cell such as a unicellular eukaryotic organism, a fungus or yeast, or a cell derived from a multicellular organism, e.g. an animal or a plant. It is especially in cases where glycosylation is desired that a mammalian cell is used, although glycosylation of proteins is a rare event in prokaryotes. Normally, however, a prokaryotic cell is preferred such as a bacterium belonging to the genera Mycobacteri-
35 um, Salmonella, Pseudomonas, Bacillus and Escherichia. It is preferred that the

transformed cell is an *E. coli*, *B. subtilis*, or *M. bovis* BCG cell, and it is especially preferred that the transformed cell expresses a polypeptide according of the invention. The latter opens for the possibility to produce the polypeptide of the invention by simply recovering it from the culture containing the transformed cell. In the most preferred
5 embodiment of this part of the invention the transformed cell is *Mycobacterium bovis* BCG strain: Danish 1331, which is the *Mycobacterium bovis* strain Copenhagen from the Copenhagen BCG Laboratory, Statens Seruminstitut, Denmark.

Example

Example 1: Cloning and expression of Rv0284, Rv0285 and Rv3878

The coding region of Rv0285, Rv3878 and the 3'-part (380 bp) of Rv0284 were amplified by PCR using following primer sets:

5 **Rv0284-F:** CTG AGA TCT CAG GTA CCG GAT TCG CCG
*Bgl*II

Rv0284-R: CTC CCA TGG TCA TGA CTG ACT CCC CTT
*Nco*I

10

Rv0285-F: CTG AGA TCT ATG ACG TTG CGA GTG GTT
*Bgl*II

Rv0285-R: CTC CCA TGG TCA GCC GCC CAC GAC CCC
15 *Nco*I

Rv3878-F: CTG AGA TCT GCT ACT GTT AAC AGA TCG
*Bgl*II

20 **Rv3878-R:** CCG CTC GAG CTA CAA CGT TGT GGT TGT
*Xho*I

PCR reactions contained 10 ng of *M. tuberculosis* H37Rv DNA in 1x low salt Taq⁺ buffer from Stratagene supplemented with 250 mM of each of the four nucleotides (Boehringer Mannheim), 0.5 mg/ml BSA (IgG technology), 1% DMSO (Merck), 5 pmoles of each
25 primer and 0.5 unit Taq⁺ DNA polymerase (Stratagene) in 10 µl reaction volume.

Reactions were initially heated to 94°C for 15 sec, followed by 30 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 90 sec, and finally by 72°C for 5 min.

The PCR fragments were cloned into the TA cloning vector pCR2.1 (Invitrogen) and then
30 transferred to the pMCT3 expression vector at the restriction sites indicated by the primers above. The resulted recombinant antigens carried 6-histidine residues at the N-terminal. All clones were confirmed by DNA sequencing.

To express his-tagged recombinant antigens, 100 ml of an overnight culture of XL-1 blue carrying the pMCT3 construct was added to 900 ml of LB-media containing 100 µg/ml

ampicillin, grown at 37°C with shaking. 1 mM IPTG was added at OD₆₀₀ = 0.4-0.6 and the culture was incubated for additional 3 - 16 hours before harvesting of cells.

For purification, the cell pellet was resuspended in 20 ml of Sonication buffer (20 mM Tris-Cl, pH 8.0, 0.5 M NaCl, 10% Glycerol, 5 mM β-ME, 0.01% Tween 20 and 1 mM imidazole). Cells were lysed and DNA was digested by treating with lysozyme (0.1 mg/ml) and DNase I (2.5 μg/ml) at room temperature for 20 min with gentle agitation. The recombinant protein was brought to solution by adding 80 ml of Sonication Buffer containing 8 M urea and sonicated the sample 5 x 30 sec, with 30 sec pausing between the pulses.

10 After centrifugation, the lysate was applied to a 5 ml TALON column (Clontech). The column was then washed with 25 ml of urea containing Sonication buffer, and the bound protein was eluted by imidazole steps (5, 10, 20, 40 and 100 mM) in the same buffer. The fractions were analyzed by silver stained SDS-PAGE, and recombinant protein containing fractions were pooled and dialyzed against 3 x 1 L of 10 mM Tris-Cl (pH 8.0), 0.15 M NaCl and 0.1% SDS. Two mg of TALON purified recombinant antigen was subjected to SDS-PAGE on an 16 x 16 cm gel. After separation, the recombinant antigen band was cut out and the protein was eluted by an Model 422 Electro-Eluter (Bio-Rad). SDS was removed from eluted protein by Chloroform/Methanol extraction.

20 **Example 2: Biological activity of the recombinant antigens.**

The purified recombinant proteins were screened for the ability to induce a T cell response measured as IFN-γ release. A preliminary screening involved testing of the IFN-γ induction of T cell lines generated from PPD positive donors. This test was followed by measuring the response in PBMC preparations obtained from TB patients, PPD positive as well as negative healthy donors.

Interferon-γ induction of T cell lines

Human donors: PBMC were obtained from healthy donors with a positive *in vitro* response to PPD.

30 **T cell line preparation:** T cell lines were prepared by culturing 5×10^6 freshly isolated PBMC/ml with viable *M. tuberculosis* at a ratio of 5 bacteria per macrophage in a total volume of 1 ml. The cells were cultured in RPMI 1640 medium (Gibco, Grand Island, N.Y) supplemented with HEPES, and 10% heat-inactivated NHS. After 7 days in culture at 37 °C and 5% CO₂, T cells were supplemented with 50 U/ml of r-IL-2 (Boehringer Mannheim)

for approximately 7 days. Finally, the T cell lines were tested for reactivity against the recombinant antigens by stimulating $1-5 \times 10^5$ cells/ml with 5 µg/ml of PPD, 3 µg/ml of rRv0284ct, 5 µg/ml of rRv0285, or 2.5 µg/ml of rRv3878 in the presence of 5×10^5 autologous antigen-presenting cells/ml. No antigen (No ag) and PHA were used as negative and positive controls, respectively. The supernatants were harvested after 4 days of culture and stored at -80 °C until the presence of IFN-γ were analysed.

Cytokine analysis: Interferon-γ (IFN-γ) was detected with a standard sandwich ELISA technique using a commercially available pair of monoclonal antibodies (Endogen, MA, US) and used according to the manufacturer's instruction. Recombinant IFN-γ (Endogen, MA, US) was used as a standard. All data are means of duplicate wells and the variation between the wells did not exceed 10 % of the mean. Responses obtained with two different T cell lines are shown in Table 1.

As shown in Table 1, high levels of IFN-γ release are observed after stimulation with the recombinant antigens ranging from 33% (rRv0284ct) to 83% (rRv3878) of the response seen after stimulation with PPD.

Table 1. Stimulation of two T cell lines with recombinant rRv0284ct, rRv0285, and rRv3878. Responses to PHA and PPD are shown for comparison. Results are presented as pg IFN-γ/ml.

T cell line						
Donor	No ag	PHA (1 µg/ml)	PPD (5 µg/ml)	rRv0284ct (3 µg/ml)	rRv0285 (5 µg/ml)	rRv3878 (2.5 µg/ml)
1	50	2975	2742	914	2019	1072
2	50	1482	803	352	548	667

Interferon-γ release from PBMC isolated from human TB patients and PPD positive and negative healthy donors

Human donors: PBMC were obtained from healthy donors with a positive *in vitro* response to purified protein derivative (PPD) or healthy donors with a negative *in vitro* response to PPD. PBMC were also obtained from TB patients with microscopy or culture proven infection. Blood samples were drawn from TB patients 0-6 months after diagnosis.

Lymphocyt preparations and cell culture: PBMC were freshly isolated by gradient centrifugation of heparinized blood on Lymphoprep (Nycomed, Oslo, Norway) and stored

in liquid nitrogen until use. The cells were resuspended in complete RPMI 1640 medium (Gibco BRL, Life Technologies) supplemented with 1% penicillin/streptomycin (Gibco BRL, Life Technologies), 1% non-essential-amino acids (FLOW, ICN Biomedicals, CA, USA), and 10% heat-inactivated normal human AB serum (NHS). The viability and number of the cells were determined by Nigrosin staining. Cell cultures were established with 1.25×10^5 PBMCs in 100 μ l in microtitre plates (Nunc, Roskilde, Denmark) and stimulated with 5 μ g/ml PPD or rRv0284ct and rRv3878 in a final concentration of 2.5 and 5 μ g/ml, respectively. No ag was used as a negative control, whereas phytohaemagglutinin (PHA) was used as a positive control. Moreover, the response to a well-known T cell antigen, ESAT-6, was included for comparison. Supernatants for the analysis of secreted cytokines were harvested after 5 days of culture, pooled, and stored at -80 °C until use.

Cytokine analysis: IFN- γ was detected as above. Responses obtained with PBMCs from 14 individual donors are shown in Table 2.

As shown in Table 2, stimulation of PBMC from TB patients as well as PPD positive donors with both rRv0284ct and rRv3878 resulted in a marked release of IFN- γ with 55% of the donors recognising the recombinant antigens at a level of more than 500 pg/ml. As expected, none of the recombinant antigens gave rise to IFN- γ release in PPD negative donors.

Table 2. Stimulation of PBMCs from 4 TB patients, 7 PPD positive healthy donors, and 3 PPD negative healthy donors with recombinant antigen. Responses to PHA, PPD, and ESAT-6 are shown for comparison. Results are given as pg IFN- γ /ml.

5 TB patients

Donor	No ag	PHA (1 μ g/ml)	PPD (5 μ g/ml)	ESAT-6 (5 μ g/ml)	rRv0284ct (2.5 μ g/ml)	rRv3878 (5 μ g/ml)
1	3	4541	4074	2154	809	3
2	92	3408	4891	611	236	2029
3	5	5282	4647	2827	308	149
4	10	4531	2077	38	140	287

PPD positive healthy donors

Donor	No ag	PHA (1 μ g/ml)	PPD (5 μ g/ml)	ESAT-6 (5 μ g/ml)	rRv0284ct (2.5 μ g/ml)	rRv3878 (5 μ g/ml)
1	74	5413	3339	0	382	77
2	14	5614	3852	198	1324	633
3	7	6165	5808	4	2951	2732
4	63	6532	6314	1567	3009	3482
5	43	4733	6195	1272	5166	2589
6	5	3809	2582	15	5	71
7	31	6716	2275	424	1449	832

PPD negative healthy donors

Donor	No ag	PHA (1 μ g/ml)	PPD (5 μ g/ml)	ESAT-6 (5 μ g/ml)	rRv0284ct (2.5 μ g/ml)	rRV3878 (5 μ g/ml)
1	0	3354	113	0	269	17
2	0	3803	563	0	22	0
3	0	3446	525	10	203	34

10

Together these analyses using T cell lines and PBMC, respectively, indicate that rRv0284ct, rRv0285, and rRv3878 are highly biologically active and recognised by PPD positive donors and TB patients.

15

Skin test reaction in TB infected guinea pigs

The skin test reactivity of the recombinant antigens was tested in *M. tuberculosis* infected guinea pigs. A group of 5 female outbreed guinea pigs of the Dunkin Hartley strain (Møllegaard Breeding and Research Center A/S, Lille Skensved, Denmark) were infected by the aerosol route in an exposure chamber of a Glas-Col® Inhalation Exposure System, which was calibrated to deliver approximately 20-25 *M. tuberculosis* Erdman bacilli into the lungs of each animal. As a control, the skin test reactivity of uninfected guinea pigs

20

was tested. Skin tests were performed 28 days after infection with injection of 5 µg of rRv0284ct, rRv0285, or rRv3878. As a positive control, the guinea pigs were sensitised with 10 tuberculin units (TU) of PPD (1TU = 0.02 µg) whereas injection of phosphate-buffered saline (PBS) was used as a negative control. Skin test responses (diameter of erythema) were read 24 h later by two experienced examinations and the results were expressed as the mean of the two readings. The variation between the two readings was less than 10%. Skin test responses larger than 5 mm were regarded as positive.

As seen in Table 3, injection of rRv3878 induced a marked Delayed Type Hypersensitivity (DTH) reaction at the same level as after injection with PPD. rRv0284ct and rRv0285 resulted in a highly significant DTH reaction ($P < 0.005$; Tukey test). As expected, none of the antigens induced non-specific response in uninfected guinea pigs (Table 4).

Table 3. DTH erythema diameter (shown in mm) in guinea pigs aerosol infected with *M. tuberculosis* after stimulation with recombinant antigens.

Antigen ^a	Skin reaction (mm) ^b	SEM
PBS	3.10	0.30
PPD	13.10	1.18
rRv0284ct	8.40	0.45
rRv0285	7.00	1.08
rRv3878	14.56	1.05

^a The recombinant antigens were tested in a concentration of 5 µg, whereas 10 TU of PPD were used.

^b The skin reactions are measured in mm erythema 24 h after intradermal injection. The values are the mean of erythema diameter of five animals and the SEM are indicated. The values for rRv3878 are the mean of four animals.

Table 4. DTH erythema diameter (shown in mm) in non-infected guinea pigs after stimulation with recombinant antigens.

Antigen ^a	Skin reaction (mm) ^b	SEM
PBS	2.60	0.36
PPD	3.00	0.44
rRv0284ct	2.5	0.18
rRv0285	3.45	0.74
rRv3878	2.5	0.18

^a The recombinant antigens were tested in a concentration of 5 µg, whereas 10 TU of PPD were used.

^b The skin reactions are measured in mm erythema 24 h after intradermal injection. The values are the mean of erythema diameter of five animals and the SEM are indicated.

Claims

1. A substantially pure polypeptide fragment which comprises an amino acid sequence as shown in SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6 or SEQ ID NO: 8 or comprises an amino acid sequence analogue having a sequence identity with any of said polypeptide sequences of at least 70% and at the same time being immunologically equivalent to said polypeptide sequence.
2. A substantially pure polypeptide fragment which comprises a T-cell epitope of the polypeptide sequence as shown in SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6 or SEQ ID NO: 8 and at the same time being immunologically equivalent to said polypeptide.
3. A fusion polypeptide comprising at least one polypeptide fragment according to any of the preceding claims and at least one fusion partner.
4. A fusion polypeptide according to claim 3, wherein the fusion partner is selected from the group consisting of a polypeptide fragment derived from a bacterium belonging to the tuberculosis complex, such as ESAT-6 or at least one T-cell epitope thereof, MPB64 or at least one T-cell epitope thereof, MPT64 or at least one T-cell epitope thereof, and MPB59 or at least one T-cell epitope thereof.
5. A polypeptide according to any of the preceding claims which is lipidated so as to allow a self-adjuvating effect of the polypeptide.
6. A substantially pure polypeptide according to any of the preceding claims for use as a pharmaceutical.
7. Use of a substantially pure polypeptide according to any of the preceding claims for the preparation of a pharmaceutical composition for the diagnosis of tuberculosis caused by *Mycobacterium tuberculosis*, *Mycobacterium africanum* or *Mycobacterium bovis*.
8. Use of a substantially pure polypeptide according to any of the preceding claims for the preparation of a pharmaceutical composition for the vaccination against infections caused by *Mycobacterium tuberculosis*, *Mycobacterium africanum* or *Mycobacterium bovis*.

9. An immunologic composition comprising a polypeptide according to any of the preceding claims.
- 5 10. An immunologic composition, which is in the form of a vaccine.
11. An immunologic composition, which is in the form of a skin test reagent.
12. A nucleic acid fragment in isolated form which
- 10 1) comprises a nucleic acid sequence which encodes a polypeptide as defined in any of claims 1-5, or comprises a nucleic acid sequence complementary thereto; or
- 2) has a length of at least 10 nucleotides and hybridizes readily under stringent
- 15 hybridization conditions with a nucleic acid fragment which has a nucleotide sequence selected from
- SEQ ID NO: 1 or a sequence complementary thereto,
SEQ ID NO: 3 or a sequence complementary thereto,
SEQ ID NO: 5 or a sequence complementary thereto, and
- 20 SEQ ID NO: 7 or a sequence complementary thereto.
13. A nucleic acid fragment according to claim 12, which is a DNA fragment.
14. A nucleic acid fragment according to claim 12 or 13 for use as a pharmaceutical.
- 25 15. A vaccine comprising a nucleic acid fragment according to claim 12 or 13, the vaccine effecting *in vivo* expression of antigen by an animal, including a human being, to whom the vaccine has been administered, the amount of expressed antigen being effective to confer substantially increased resistance to infections with mycobacteria of the tuberculo-
- 30 sis complex in an animal, including a human being.
16. Use of a nucleic acid fragment according to claim 12 or 13 for the preparation of a pharmaceutical composition for the diagnosis of tuberculosis caused by *Mycobacterium tuberculosis*, *Mycobacterium africanum* or *Mycobacterium bovis*.

17. Use of a nucleic acid fragment according to claim 12 or 13 for the preparation of a pharmaceutical composition for the vaccination against tuberculosis caused by *Mycobacterium tuberculosis*, *Mycobacterium africanum* or *Mycobacterium bovis*.
- 5 18. A vaccine for immunizing an animal, including a human being, against tuberculosis caused by mycobacteria belonging to the tuberculosis complex, comprising as the effective component a non-pathogenic microorganism, wherein at least one copy of a DNA fragment comprising a DNA sequence encoding a polypeptide according to any of claims 1-5 has been incorporated into the genome of the microorganism in a manner
10 allowing the microorganism to express and optionally secrete the polypeptide.
19. A replicable expression vector which comprises a nucleic acid fragment according to claim 12 or 13.
- 15 20. A transformed cell harbouring at least one vector according to claim 19.
21. A method for producing a polypeptide according to any of claims 1-5, comprising
inserting a nucleic acid fragment according to claim 12 or 13 into a vector which is able to
20 replicate in a host cell, introducing the resulting recombinant vector into the host cell, culturing the host cell in a culture medium under conditions sufficient to effect expression of the polypeptide, and recovering the polypeptide from the host cell or culture medium; or
isolating the polypeptide from whole mycobacteria of the tuberculosis complex or from
25 lysates or fractions thereof; or
synthesizing the polypeptide by solid or liquid phase peptide synthesis.
22. A method of diagnosing tuberculosis caused by *Mycobacterium tuberculosis*,
30 *Mycobacterium africanum* or *Mycobacterium bovis* in an animal, including a human being, comprising intradermally injecting, in the animal, a polypeptide according to any of claims 1-5 or an immunologic composition according to claim 9, a positive skin response at the location of injection being indicative of the animal having tuberculosis, and a negative skin response at the location of injection being indicative of the animal not having tuberculosis.

23. A method for immunising an animal, including a human being, against tuberculosis caused by mycobacteria belonging to the tuberculosis complex, comprising administering to the animal the polypeptide according to any of claims 1-5, the immunologic composition according to claim 9, or the vaccine according to claim 18.

5

24. A monoclonal or polyclonal antibody, which is specifically reacting with a polypeptide according to any of claims 1-5 in an immuno assay, or a specific binding fragment of said antibody.

SEQUENCE LISTING

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ctg gcc aac ctc atc gag gcg cgc cgg ccg ccg gcc ggg atg tct gcg Leu Ala Asn Leu Ile Glu Ala Arg Arg Pro Pro Ala Gly Met Ser Ala 1170 1175 1180			3552
gcc gag ctg tcc cgc tgg acc ttt gcc ggg cac acc cac tac ctg atc Ala Glu Leu Ser Arg Trp Thr Phe Ala Gly His Thr His Tyr Leu Ile 1185 1190 1195 1200			3600
atc gac gac gtc gac cag gta ccg gat tcc ccg gcg atg acc ggt ccc Ile Asp Asp Val Asp Gln Val Pro Asp Ser Pro Ala Met Thr Gly Pro 1205 1210 1215			3648
tac atc gga cag cgg ccg tgg acc ccg ctg atc ggt ctc ctg gcc cag Tyr Ile Gly Gln Arg Pro Trp Thr Pro Leu Ile Gly Leu Leu Ala Gln 1220 1225 1230			3696
gcc ggc gac ttg ggg cta cgg gtg att gtc acc ggg cgt gcc act gga Ala Gly Asp Leu Gly Leu Arg Val Ile Val Thr Gly Arg Ala Thr Gly 1235 1240 1245			3744
tcc gcg cac ctg ctg atg aca agt ccg ttg ctg cgc cgg ttc aac gac Ser Ala His Leu Leu Met Thr Ser Pro Leu Leu Arg Arg Phe Asn Asp 1250 1255 1260			3792
ctg cag gcg acc acg ctg atg ttg gca ggc aat ccg gcc gac agc ggc Leu Gln Ala Thr Thr Leu Met Leu Ala Gly Asn Pro Ala Asp Ser Gly 1265 1270 1275 1280			3840
aag att cgc ggt gag cgg ttt gcc cga ttg cct gct gga cga gca att Lys Ile Arg Gly Glu Arg Phe Ala Arg Leu Pro Ala Gly Arg Ala Ile 1285 1290 1295			3888
ctg ttg acc gac agt gat agt cca acc tac gtg cag ttg atc aac ccg Leu Leu Thr Asp Ser Asp Ser Pro Thr Tyr Val Gln Leu Ile Asn Pro 1300 1305 1310			3936
ctg gtc gat gcg gcc gcg gtt tct ggt gaa acc caa cag aag ggg agt			3984

Leu Val Asp Ala Ala Ala Val Ser Gly Glu Thr Gln Gln Lys Gly Ser
 1315 1320 1325

cag tca
 Gln Ser
 1330

3990

<210> 2
 <211> 1330
 <212> PRT
 <213> Mycobacterium Tuberculosis

<400> 2

Val Ser Arg Leu Ile Phe Glu Ala Arg Arg Arg Leu Ala Pro Pro Ser
 1 5 10 15
 Ser His Gln Gly Thr Ile Ile Ile Glu Ala Pro Pro Glu Leu Pro Arg
 20 25 30
 Val Ile Pro Pro Ser Leu Leu Arg Arg Ala Leu Pro Tyr Leu Ile Gly
 35 40 45
 Ile Leu Ile Val Gly Met Ile Val Ala Leu Val Ala Thr Gly Met Arg
 50 55 60
 Val Ile Ser Pro Gln Thr Leu Phe Phe Pro Phe Val Leu Leu Leu Ala
 65 70 75 80
 Ala Thr Ala Leu Tyr Arg Gly Asn Asp Lys Lys Met Arg Thr Glu Glu
 85 90 95
 Val Asp Ala Glu Arg Ala Asp Tyr Leu Arg Tyr Leu Ser Val Val Arg
 100 105 110
 Asp Asn Ile Arg Ala Gln Ala Ala Glu Gln Arg Ala Ser Ala Leu Trp
 115 120 125
 Ser His Pro Asp Pro Thr Ala Leu Ala Ser Val Pro Gly Ser Arg Arg
 130 135 140
 Gln Trp Glu Arg Asp Pro His Asp Pro Asp Phe Leu Val Leu Arg Ala
 145 150 155 160
 Gly Arg His Thr Val Pro Leu Ala Thr Thr Leu Arg Val Asn Asp Thr
 165 170 175
 Ala Asp Glu Ile Asp Leu Glu Pro Val Ser His Ser Ala Leu Arg Ser
 180 185 190
 Leu Leu Asp Thr Gln Arg Ser Ile Gly Asp Val Pro Thr Gly Ile Asp
 195 200 205
 Leu Thr Lys Val Ser Pro Ile Thr Val Leu Gly Glu Arg Ala Gln Val
 210 215 220
 Arg Ala Val Leu Arg Ala Trp Ile Ala Gln Ala Val Thr Trp His Asp
 225 230 235 240
 Pro Thr Val Leu Gly Val Ala Leu Ala Ala Arg Asp Leu Glu Gly Arg
 245 250 255
 Asp Trp Asn Trp Leu Lys Trp Leu Pro His Val Asp Ile Pro Gly Arg
 260 265 270
 Leu Asp Ala Leu Gly Pro Ala Arg Asn Leu Ser Thr Asp Pro Asp Glu
 275 280 285
 Leu Ile Ala Leu Leu Gly Pro Val Leu Ala Asp Arg Pro Ala Phe Thr
 290 295 300
 Gly Gln Pro Thr Asp Ala Leu Arg His Leu Leu Ile Val Val Asp Asp
 305 310 315 320
 Pro Asp Tyr Asp Leu Gly Ala Ser Pro Leu Ala Val Gly Arg Ala Gly
 325 330 335
 Val Thr Val Val His Cys Ser Ala Ser Ala Pro His Arg Glu Gln Tyr
 340 345 350
 Ser Asp Pro Glu Lys Pro Ile Leu Arg Val Ala His Gly Ala Ile Glu

		355					360					365				
Arg	Trp	Gln	Thr	Gly	Gly	Trp	Gln	Pro	Tyr	Ile	Asp	Ala	Ala	Asp	Gln	
	370					375					380					
Phe	Ser	Ala	Asp	Glu	Ala	Ala	His	Leu	Ala	Arg	Arg	Leu	Ser	Arg	Trp	
385					390					395					400	
Asp	Ser	Asn	Pro	Thr	His	Ala	Gly	Leu	Arg	Ser	Ala	Ala	Thr	Arg	Gly	
				405					410					415		
Ala	Ser	Phe	Thr	Thr	Leu	Leu	Gly	Ile	Glu	Asp	Ala	Ser	Arg	Leu	Asp	
			420					425					430			
Val	Pro	Ala	Leu	Trp	Ala	Pro	Arg	Arg	Arg	Asp	Glu	Glu	Leu	Arg	Val	
		435					440					445				
Pro	Ile	Gly	Val	Thr	Gly	Thr	Gly	Glu	Pro	Leu	Met	Phe	Asp	Leu	Lys	
	450				455						460					
Asp	Glu	Ala	Glu	Gly	Gly	Met	Gly	Pro	His	Gly	Leu	Met	Ile	Gly	Met	
465					470					475					480	
Thr	Gly	Ser	Gly	Lys	Ser	Gln	Thr	Leu	Met	Ser	Ile	Leu	Leu	Ser	Leu	
				485					490					495		
Leu	Thr	Thr	His	Ser	Ala	Glu	Arg	Leu	Ile	Val	Ile	Tyr	Ala	Asp	Phe	
			500					505					510			
Lys	Gly	Glu	Ala	Gly	Ala	Asp	Ser	Phe	Arg	Asp	Phe	Pro	Gln	Val	Val	
		515					520					525				
Ala	Val	Ile	Ser	Asn	Met	Ala	Glu	Lys	Lys	Ser	Leu	Ala	Asp	Arg	Phe	
	530					535					540					
Ala	Asp	Thr	Leu	Arg	Gly	Glu	Val	Ala	Arg	Arg	Glu	Met	Leu	Leu	Arg	
545					550					555					560	
Glu	Ala	Gly	Arg	Lys	Val	Gln	Gly	Ser	Ala	Phe	Asn	Ser	Val	Leu	Glu	
				565					570					575		
Tyr	Glu	Asn	Ala	Ile	Ala	Ala	Gly	His	Ser	Leu	Pro	Pro	Ile	Pro	Thr	
			580					585					590			
Leu	Phe	Val	Val	Ala	Asp	Glu	Phe	Thr	Leu	Met	Leu	Ala	Asp	His	Pro	
		595					600					605				
Glu	Tyr	Ala	Glu	Leu	Phe	Asp	Tyr	Val	Ala	Arg	Lys	Gly	Arg	Ser	Phe	
	610					615					620					
Arg	Ile	His	Ile	Leu	Phe	Ala	Ser	Gln	Thr	Leu	Asp	Val	Gly	Lys	Ile	
625					630					635					640	
Lys	Asp	Ile	Asp	Lys	Asn	Thr	Ala	Tyr	Arg	Ile	Gly	Leu	Lys	Val	Ala	
				645					650					655		
Ser	Pro	Ser	Val	Ser	Arg	Gln	Ile	Ile	Gly	Val	Glu	Asp	Ala	Tyr	His	
			660					665					670			
Ile	Glu	Ser	Gly	Lys	Glu	His	Lys	Gly	Val	Gly	Phe	Leu	Val	Pro	Ala	
		675					680					685				
Pro	Gly	Ala	Thr	Pro	Ile	Arg	Phe	Arg	Ser	Thr	Tyr	Val	Asp	Gly	Ile	
	690					695					700					
Tyr	Glu	Pro	Pro	Gln	Thr	Ala	Lys	Ala	Val	Val	Val	Gln	Ser	Val	Pro	
705					710											

Ser	Gly	Lys	Ser	Thr	Ala	Leu	Gln	Thr	Phe	Ile	Leu	Ser	Ala	Ala	Ser	835	840	845
Leu	His	Ser	Pro	His	Glu	Val	Ser	Phe	Tyr	Cys	Leu	Asp	Tyr	Gly	Gly	850	855	860
Gly	Gln	Leu	Arg	Ala	Leu	Gln	Asp	Leu	Ala	His	Val	Gly	Ser	Val	Ala	865	870	875
Ser	Ala	Leu	Glu	Pro	Glu	Arg	Ile	Arg	Arg	Thr	Phe	Gly	Glu	Leu	Glu	885	890	895
Gln	Leu	Leu	Leu	Ser	Arg	Gln	Gln	Arg	Glu	Val	Phe	Arg	Asp	Arg	Gly	900	905	910
Ala	Asn	Gly	Ser	Thr	Pro	Asp	Asp	Gly	Phe	Gly	Glu	Val	Phe	Leu	Val	915	920	925
Ile	Asp	Asn	Leu	Tyr	Gly	Phe	Gly	Arg	Asp	Asn	Thr	Asp	Gln	Phe	Asn	930	935	940
Thr	Arg	Asn	Pro	Leu	Leu	Ala	Arg	Val	Thr	Glu	Leu	Val	Asn	Val	Gly	945	950	955
Leu	Ala	Tyr	Gly	Ile	His	Val	Ile	Ile	Thr	Thr	Pro	Ser	Trp	Leu	Glu	965	970	975
Val	Pro	Leu	Ala	Met	Arg	Asp	Gly	Leu	Gly	Leu	Arg	Leu	Glu	Leu	Arg	980	985	990
Leu	His	Asp	Ala	Arg	Asp	Ser	Asn	Val	Arg	Val	Val	Gly	Ala	Leu	Arg	995	1000	1005
Arg	Pro	Ala	Asp	Ala	Val	Pro	His	Asp	Gln	Pro	Gly	Arg	Gly	Leu	Thr	1010	1015	1020
Met	Ala	Ala	Glu	His	Phe	Leu	Phe	Ala	Ala	Pro	Glu	Leu	Asp	Ala	Gln	1025	1030	1035
Thr	Asn	Pro	Val	Ala	Ala	Ile	Asn	Ala	Arg	Tyr	Pro	Gly	Met	Ala	Ala	1045	1050	1055
Pro	Pro	Val	Arg	Leu	Leu	Pro	Thr	Asn	Leu	Ala	Pro	His	Ala	Val	Gly	1060	1065	1070
Glu	Leu	Tyr	Arg	Gly	Pro	Asp	Gln	Leu	Val	Ile	Gly	Gln	Arg	Glu	Glu	1075	1080	1085
Asp	Leu	Ala	Pro	Val	Ile	Leu	Asp	Leu	Ala	Ala	Asn	Pro	Leu	Leu	Met	1090	1095	1100
Val	Phe	Gly	Asp	Ala	Arg	Ser	Gly	Lys	Thr	Thr	Leu	Leu	Arg	His	Ile	1105	1110	1115
Ile	Arg	Thr	Val	Arg	Glu	His	Ser	Thr	Ala	Asp	Arg	Val	Ala	Phe	Thr	1125	1130	1135
Val	Leu	Asp	Arg	Arg	Leu	His	Leu	Val	Asp	Glu	Pro	Leu	Phe	Pro	Asp	1140	1145	1150
Asn	Glu	Tyr	Thr	Ala	Asn	Ile	Asp	Arg	Ile	Ile	Pro	Ala	Met	Leu	Gly	1155	1160	1165
Leu	Ala	Asn	Leu	Ile	Glu	Ala	Arg	Arg	Pro	Pro	Ala	Gly	Met	Ser	Ala	1170	1175	1180
Ala	Glu	Leu	Ser	Arg	Trp	Thr	Phe	Ala	Gly	His	Thr	His	Tyr	Leu	Ile	1185	1190	1195
Ile	Asp	Asp	Val	Asp	Gln	Val	Pro	Asp	Ser	Pro	Ala	Met	Thr	Gly	Pro	1205	1210	1215
Tyr	Ile	Gly	Gln	Arg	Pro	Trp	Thr	Pro	Leu	Ile	Gly	Leu	Leu	Ala	Gln	1220	1225	1230
Ala	Gly	Asp	Leu	Gly	Leu	Arg	Val	Ile	Val	Thr	Gly	Arg	Ala	Thr	Gly	1235	1240	1245
Ser	Ala	His	Leu	Leu	Met	Thr	Ser	Pro	Leu	Leu	Arg	Arg	Phe	Asn	Asp	1250	1255	1260
Leu	Gln	Ala	Thr	Thr	Leu	Met	Leu	Ala	Gly	Asn	Pro	Ala	Asp	Ser	Gly	1265	1270	1275
Lys	Ile	Arg	Gly	Glu	Arg	Phe	Ala	Arg	Leu	Pro	Ala	Gly	Arg	Ala	Ile	1285	1290	1295
Leu	Leu	Thr	Asp	Ser	Asp	Ser	Pro	Thr	Tyr	Val	Gln	Leu	Ile	Asn	Pro			

10

1300 1305 1310
 Leu Val Asp Ala Ala Ala Val Ser Gly Glu Thr Gln Gln Lys Gly Ser
 1315 1320 1325
 Gln Ser
 1330

<210> 3
 <211> 375
 <212> DNA
 <213> Mycobacterium Tuberculosis

<220>
 <221> CDS
 <222> (1)...(375)

<400> 3
 cag gta ccg gat tcg ccg gcg atg acc ggt ccc tac atc gga cag cgg 48
 Gln Val Pro Asp Ser Pro Ala Met Thr Gly Pro Tyr Ile Gly Gln Arg
 1 5 10 15
 ccg tgg acc ccg ctg atc ggt ctc ctg gcc cag gcc ggc gac ttg ggg 96
 Pro Trp Thr Pro Leu Ile Gly Leu Leu Ala Gln Ala Gly Asp Leu Gly
 20 25 30
 cta cgg gtg att gtc acc ggg cgt gcc act gga tcg gcg cac ctg ctg 144
 Leu Arg Val Ile Val Thr Gly Arg Ala Thr Gly Ser Ala His Leu Leu
 35 40 45
 atg aca agt ccg ttg ctg cgc cgg ttc aac gac ctg cag gcg acc acg 192
 Met Thr Ser Pro Leu Leu Arg Arg Phe Asn Asp Leu Gln Ala Thr Thr
 50 55 60
 ctg atg ttg gca ggc aat ccg gcc gac agc ggc aag att cgc ggt gag 240
 Leu Met Leu Ala Gly Asn Pro Ala Asp Ser Gly Lys Ile Arg Gly Glu
 65 70 75 80
 cgg ttt gcc cga ttg cct gct gga cga gca att ctg ttg acc gac agt 288
 Arg Phe Ala Arg Leu Pro Ala Gly Arg Ala Ile Leu Leu Thr Asp Ser
 85 90 95
 gat agt cca acc tac gtg cag ttg atc aac ccg ctg gtc gat gcg gcc 336
 Asp Ser Pro Thr Tyr Val Gln Leu Ile Asn Pro Leu Val Asp Ala Ala
 100 105 110
 gcg gtt tct ggt gaa acc caa cag aag ggg agt cag tca 375
 Ala Val Ser Gly Glu Thr Gln Gln Lys Gly Ser Gln Ser
 115 120 125

<210> 4
 <211> 125
 <212> PRT
 <213> Mycobacterium Tuberculosis

<400> 4
 Gln Val Pro Asp Ser Pro Ala Met Thr Gly Pro Tyr Ile Gly Gln Arg
 1 5 10 15
 Pro Trp Thr Pro Leu Ile Gly Leu Leu Ala Gln Ala Gly Asp Leu Gly
 20 25 30

```

Leu Arg Val Ile Val Thr Gly Arg Ala Thr Gly Ser Ala His Leu Leu
  35          40          45
Met Thr Ser Pro Leu Leu Arg Arg Phe Asn Asp Leu Gln Ala Thr Thr
  50          55          60
Leu Met Leu Ala Gly Asn Pro Ala Asp Ser Gly Lys Ile Arg Gly Glu
  65          70          75          80
Arg Phe Ala Arg Leu Pro Ala Gly Arg Ala Ile Leu Leu Thr Asp Ser
      85          90          95
Asp Ser Pro Thr Tyr Val Gln Leu Ile Asn Pro Leu Val Asp Ala Ala
      100          105          110
Ala Val Ser Gly Glu Thr Gln Gln Lys Gly Ser Gln Ser
      115          120          125

```

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<210> 5
<211> 306
<212> DNA
<213> Mycobacterium Tuberculosis

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<220>
<221> CDS
<222> (1)...(306)

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<400> 5
atg acg ttg cga gtg gtt ccg gag ggg ctg gcc gca gcc agc gct gcg      48
Met Thr Leu Arg Val Val Pro Glu Gly Leu Ala Ala Ala Ser Ala Ala
  1          5          10          15

gtg gaa gcg ctg acg gcg cgg ttg gcc gcc gcg cat gcg agc gca gcg      96
Val Glu Ala Leu Thr Ala Arg Leu Ala Ala Ala His Ala Ser Ala Ala
      20          25          30

ccg gtg att acc gcg gta gtg ccg ccg gcg gcg gat ccg gtg tcg ctg      144
Pro Val Ile Thr Ala Val Val Pro Pro Ala Ala Asp Pro Val Ser Leu
      35          40          45

cag acc gcg gcc ggg ttc agt gca cag ggc gtc gag cac gcg gtc gtc      192
Gln Thr Ala Ala Gly Phe Ser Ala Gln Gly Val Glu His Ala Val Val
      50          55          60

acc gcc gaa ggt gtc gaa gag ctg gga cgc gcc ggc gtt ggt gtg ggc      240
Thr Ala Glu Gly Val Glu Glu Leu Gly Arg Ala Gly Val Gly Val Gly
      65          70          75          80

gaa tcc ggc gcc agc tac ctg gcc ggt gat gcg gcc gcc gcc gct acg      288
Glu Ser Gly Ala Ser Tyr Leu Ala Gly Asp Ala Ala Ala Ala Ala Thr
      85          90          95

tac ggg gtc gtg ggc ggc
Tyr Gly Val Val Gly Gly
      100

```

```

<210> 6
<211> 102
<212> PRT
<213> Mycobacterium Tuberculosis

```

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<400> 6
Met Thr Leu Arg Val Val Pro Glu Gly Leu Ala Ala Ala Ser Ala Ala

```

12

1	5	10	15
Val Glu Ala	Leu Thr Ala Arg Leu	Ala Ala His Ala	Ser Ala Ala
20	25	30	
Pro Val Ile	Thr Ala Val Val	Pro Ala Ala Asp	Pro Val Ser Leu
35	40	45	
Gln Thr Ala	Ala Gly Phe Ser	Ala Gln Gly Val	Glu His Ala Val Val
50	55	60	
Thr Ala Glu	Gly Val Glu Glu Leu	Gly Arg Ala Gly	Val Gly Val Gly
65	70	75	80
Glu Ser Gly	Ala Ser Tyr Leu	Ala Gly Asp Ala	Ala Ala Ala Thr
85	90	95	
Tyr Gly Val	Val Gly Gly		
100			

<210> 7
 <211> 840
 <212> DNA
 <213> Mycobacterium Tuberculosis

<220>
 <221> CDS
 <222> (1)...(840)

<400> 7	
atg gct gaa ccg ttg gcc gtc gat ccc acc ggc ttg agc gca gcg gcc	48
Met Ala Glu Pro Leu Ala Val Asp Pro Thr Gly Leu Ser Ala Ala Ala	
1 5 10 15	
gcg aaa ttg gcc ggc ctc gtt ttt ccg cag cct ccg gcg ccg atc gcg	96
Ala Lys Leu Ala Gly Leu Val Phe Pro Gln Pro Pro Ala Pro Ile Ala	
20 25 30	
gtc agc gga acg gat tcg gtg gta gca gca atc aac gag acc atg cca	144
Val Ser Gly Thr Asp Ser Val Val Ala Ala Ile Asn Glu Thr Met Pro	
35 40 45	
agc atc gaa tcg ctg gtc agt gac ggg ctg ccc ggc gtg aaa gcc gcc	192
Ser Ile Glu Ser Leu Val Ser Asp Gly Leu Pro Gly Val Lys Ala Ala	
50 55 60	
ctg act cga aca gca tcc aac atg aac gcg gcg gcg gac gtc tat gcg	240
Leu Thr Arg Thr Ala Ser Asn Met Asn Ala Ala Ala Asp Val Tyr Ala	
65 70 75 80	
aag acc gat cag tca ctg gga acc agt ttg agc cag tat gca ttc ggc	288
Lys Thr Asp Gln Ser Leu Gly Thr Ser Leu Ser Gln Tyr Ala Phe Gly	
85 90 95	
tcg tcg ggc gaa ggc ctg gct ggc gtc gcc tcg gtc ggt ggt cag cca	336
Ser Ser Gly Glu Gly Leu Ala Gly Val Ala Ser Val Gly Gly Gln Pro	
100 105 110	
agt cag gct acc cag ctg ctg agc aca ccc gtg tca cag gtc acg acc	384
Ser Gln Ala Thr Gln Leu Leu Ser Thr Pro Val Ser Gln Val Thr Thr	
115 120 125	
cag ctc ggc gag acg gcc gct gag ctg gca ccc cgt gtt gtt gcg acg	432
Gln Leu Gly Glu Thr Ala Ala Glu Leu Ala Pro Arg Val Val Ala Thr	
130 135 140	

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gtg ccg caa ctc gtt cag ctg gct ccg cac gcc gtt cag atg tcg caa      480
Val Pro Gln Leu Val Gln Leu Ala Pro His Ala Val Gln Met Ser Gln
145                      150                      155                      160

aac gca tcc ccc atc gct cag acg atc agt caa acc gcc caa cag gcc      528
Asn Ala Ser Pro Ile Ala Gln Thr Ile Ser Gln Thr Ala Gln Gln Ala
165                      170                      175

gcc cag agc gcg cag ggc ggc agc ggc cca atg ccc gca cag ctt gcc      576
Ala Gln Ser Ala Gln Gly Gly Ser Gly Pro Met Pro Ala Gln Leu Ala
180                      185

agc gct gaa aaa ccg gcc acc gag caa gcg gag ccg gtc cac gaa gtg      624
Ser Ala Glu Lys Pro Ala Thr Glu Gln Ala Glu Pro Val His Glu Val
195                      200                      205

aca aac gac gat cag ggc gac cag ggc gac gtg cag ccg gcc gag gtc      672
Thr Asn Asp Asp Gln Gly Asp Gln Gly Asp Val Gln Pro Ala Glu Val
210                      215                      220

gtt gcc gcg gca cgt gac gaa ggc gcc ggc gca tca ccg ggc cag cag      720
Val Ala Ala Ala Arg Asp Glu Gly Ala Gly Ala Ser Pro Gly Gln Gln
225                      230                      235                      240

ccc ggc ggg ggc gtt ccc gcg caa gcc atg gat acc gga gcc ggt gcc      768
Pro Gly Gly Gly Val Pro Ala Gln Ala Met Asp Thr Gly Ala Gly Ala
245                      250                      255

cgc cca gcg gcg agt ccg ctg gcg gcc ccc gtc gat ccg tcg act ccg      816
Arg Pro Ala Ala Ser Pro Leu Ala Ala Pro Val Asp Pro Ser Thr Pro
260                      265                      270

gca ccc tca aca acc aca acg ttg      840
Ala Pro Ser Thr Thr Thr Thr Leu
275                      280

```

```

<210> 8
<211> 280
<212> PRT
<213> Mycobacterium Tuberculosis

```

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<400> 8
Met Ala Glu Pro Leu Ala Val Asp Pro Thr Gly Leu Ser Ala Ala Ala
1      5      10      15
Ala Lys Leu Ala Gly Leu Val Phe Pro Gln Pro Pro Ala Pro Ile Ala
20      25      30
Val Ser Gly Thr Asp Ser Val Val Ala Ala Ile Asn Glu Thr Met Pro
35      40      45
Ser Ile Glu Ser Leu Val Ser Asp Gly Leu Pro Gly Val Lys Ala Ala
50      55      60
Leu Thr Arg Thr Ala Ser Asn Met Asn Ala Ala Asp Val Tyr Ala
65      70      75      80
Lys Thr Asp Gln Ser Leu Gly Thr Ser Leu Ser Gln Tyr Ala Phe Gly
85      90      95
Ser Ser Gly Glu Gly Leu Ala Gly Val Ala Ser Val Gly Gly Gln Pro
100     105     110
Ser Gln Ala Thr Gln Leu Leu Ser Thr Pro Val Ser Gln Val Thr Thr

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